DRAFT: August 31, 1994

DECISION DOCUMENT
TSCA SECTION 5(H)(4) EXEMPTION FOR
ESCHERICHIA COLI K-12

I. SUMMARY

Escherichia coli is one of a number of microorganisms which are normal inhabitants of the colons of virtually all warm-blooded mammals. *E. coli* belongs to the taxonomic family known as *Enterobacteriaceae*, which is one of the best-defined groups of bacteria. The strain *E. coli* K-12 is a debilitated strain which does not normally colonize the human intestine. It has also been shown to survive poorly in the environment, has a history of safe commercial use, and is not known to have adverse effects on microorganisms or plants. Because of its wide use as a model organism in research in microbial genetics and physiology, and its use in industrial applications, *E. coli* K-12 is one of the most extensively studied microorganisms.

II. BACKGROUND

A. Introduction

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA is proposing expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that would be exempt meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

B. Criteria for Minimizing Release from Manufacturing Facilities

The standards prescribed for the Tier I exemption require the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is proposing, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's proposed standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the bacteria used in fermentation processes are usually debilitated, either intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is proposing that the number of microorganisms in fermentor gases be reduced by at least two logs prior to the gases being exhausted from the fermentor. EPA selected this number based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted in a gas from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also proposing that the requirements specify that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most

individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that entry be limited to essential personnel also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

C. Introduced Genetic Material Criteria

In order to qualify for either Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for metabolizing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms proposed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should not prevent most microorganisms from meeting the exemption criteria, since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only if the introduced genetic material has been altered or selected to enhance frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Escherichia coli K-12, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of E. coli K-12 will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of E. coli K-12, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of E. coli K-12, and EPA's review of the conditions selected.

D. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Escherichia coli K-12 is discussed in the next unit.

III. EVALUATION OF ESCHERICHIA COLI K-12

A. History of Use

1. History of safe commercial use. E. coli K-12 has a history of safe use. Its derivatives are currently used in a large number of industrial applications, including the production of specialty chemicals (e.g., L-aspartic, inosinic, and adenylic acids) and human drugs such as insulin and somatostatin. In general, E. coli K-12 is one of the most extensively studied bacteria, and has been used in genetic studies in laboratories worldwide. Experience with the use of E. coli is reflected in its classification under the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules. With the exception of strains which are known to be pathogenic, E. coli is considered a Class 1 Agent under the NIH Guidelines. Most experiments involving E. coli K-12 have been exempted, except in certain circumstances, from the NIH Guidelines based on an analysis of safety, and transfers of genetic material between species that exchange DNA by known physiological processes with the genus Escherichia are exempted.

2. Products subject to TSCA jurisdiction. E. coli is capable of producing a number of specialty chemicals, such as enzymes, which could be subject to TSCA. EPA has already reviewed under TSCA the use of E. coli as an intermediate to produce an insulin-like hormone for use in cell culture media. In 1994, EPA received a submission for use of a genetically modified strain of E. coli K-12 as an intermediate to produce indigo.

B. Identification of Microorganism

1. Classification. Escherichia coli is a member of the family Enterobacteriaceae and is a Gram-negative rod and a facultative anaerobe which commonly occurs in the digestive tract. E. coli K-12 was originally isolated from a convalescent diphtheria patient in 1922. Because it has been maintained in the laboratory for over 70 years, it is now considered an enfeebled organism, which is unable to colonize the intestines of humans and animals.

2. Related species of concern. Taxonomically, the four species of the genus Shigella are closely related to E. coli. Shigella species cause diarrhea in humans and are classified as Class 2 agents under the NIH Guidelines. The Shigella species and E. coli share a high level of DNA sequence homology and many protein and polysaccharide capsular antigens. These capsular antigens can be used to distinguish between E.

coli strains and the pattern of capsular antigens that determine the organism's "serotype". The two genera can be distinguished based on the fact that _E_. _coli_ has a unique colony morphology when grown on certain differential laboratory media. Commercially prepared kits for distinguishing between these organisms are available. K-12 strains are distinguishable from both _Shigella_ and other _Escherichia_.

C. Risk Summary

1. _Studies regarding potential for adverse effects_. There is no evidence associating adverse effects with _E_. _coli_ K-12. _E_. _coli_ K-12 is now considered an enfeebled organism as a result of being maintained in the laboratory environment for over 70 years (Williams-Smith, 1978). As a result, K-12 strains are unable to colonize the intestines of humans and other animals under normal conditions.

2. _Studies regarding survival in the environment_. Given its natural habitat of the large bowel of mammals, _E_. _coli_ will not likely survive for long periods in soil, water, or air. _E_. _coli_ K-12 has lost the ability to colonize the gut and has been shown to have poorer survival characteristics in soil and water than other _E_. _coli_. The ability of _E_. _coli_ to survive under environmental conditions is thus very limited. _E_. _coli_ K-12 has no known survival mechanisms in the environment, such as the ability to produce spores.

IV. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. _Escherichia_ _coli_ K-12 is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. In addition to assessing the risk of _E_. _coli_ K-12, EPA has developed criteria limiting the potential for transfer of and expression of toxin sequences, and the conditions of use specified in the exemption are met (Tier I) or will be reviewed by EPA to ensure adequate risk reduction (Tier II). EPA requirements for minimizing numbers of viable microorganisms emitted are within standard operating procedures for the industry, and both the procedures and the structures specified in the exemption are the type industry uses to protect their products from contamination.

The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

V. RECOMMENDATION AND RATIONALE

A. Recommendation E. coli K-12 is recommended for a TSCA section (5)(h)(4) tiered exemption.

B. Rationale

1. Risks from use of the recipient microorganism E. coli K-12 are low. There are no human health concerns related to the use of E. coli K-12 strains. In addition, E. coli K-12 is a debilitated organism not adapted for survival in the human gut or in the environment. Releases of this microorganism to the environment through fermentation uses would not pose any significant ecological hazards, because this microorganism is not expected to survive and it is not pathogenic to animals or plants.

2. Use of strains of E. coli K-12 which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption. When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

Attachment 1:

INTEGRATED RISK ASSESSMENT OF

ESCHERICHIA COLI K-12 DERIVATIVES

I. INTRODUCTION

Escherichia coli is one of a number of microorganisms which are normal inhabitants of the colons of virtually all warm-blooded mammals. *E. coli* belongs to the taxonomic family known as *Enterobacteriaceae*, which is one of the best-defined groups of bacteria. The strain *E. coli* K-12 is a debilitated strain which does not normally colonize the human intestine. It has also been shown to survive poorly in the environment, has a history of safe commercial use, and is not known to have adverse effects on microorganisms or plants. Because of its wide use as a model organism in research in microbial genetics and physiology, and its use in industrial applications, *E. coli* K-12 is one of the most extensively studied microorganisms.

History of Commercial Use and Products Subject to TSCA Jurisdiction

E. coli K-12 has a history of safe use. Its derivatives are currently used in a large number of industrial applications, including the production of specialty chemicals (e.g., L-aspartic, inosinic, and adenylic acids) and human drugs such as insulin and somatostatin (Dynamac, 1990). Further, *E. coli* can produce a number of specialty chemicals such as enzymes which would be regulated under TSCA. An insulin-like hormone for use as a component of cell culture media, resulting from a fermentation application in which *E. coli* was used as the recipient, has already been reviewed under TSCA (Premanufacture Notice P87-693). EPA recently reviewed a submission (94-1558) for use of E. coli K-12 to produce indigo for use as a dye. In general, *E. coli* K-12 is one of the most extensively studied bacteria, and has been used in genetic studies in laboratories worldwide.

Experience with the use of *E. coli* is reflected in its classification under the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules ("NIH Guidelines"; USDepartment of Health and Human Services, 1986). With the exception of strains which are known to be pathogenic, *E. coli* is considered a Class 1 Agent under the NIH Guidelines. Class 1 consists of all organisms which are not either human or animal pathogens. Most experiments involving *E. coli* K-12 have been exempted from the NIH Guidelines based on an analysis of safety, except in certain circumstances (see Appendix C-II of the NIH Guidelines). Moreover NIH, under section III-D-4 of the NIH Guidelines, exempts transfers of genetic material between species that exchange DNA by known physiological processes with the genus *Escherichia*. Included in this exemption are exchanges between *Escherichia* and the closely related genera of *Shigella*, *Salmonella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Erwinia*, *Pseudomonas aeruginosa*; also included are the species *Pseudomonas putida*, *Pseudomonas fluorescens*, *Serratia marcescens*, and *Yersinia enterocolitica*.

II. IDENTIFICATION AND TAXONOMY

A. Overview

E. *coli* belongs to the family *Enterobacteriaceae*. All *Enterobacteriaceae* are defined as gram-negative, non spore-forming rods that are facultative anaerobes. During the 1960's and 1970's, large amounts of information were generated regarding the phenotypic characteristics of the *Enterobacteriaceae*. The reasons for this increase in knowledge were two-fold. First, beginning in the early 1970's, a number of methods became available for the identification of enteric bacteria. These methods were based on biochemical or phenotypic reactions and could be performed with minimum labor and cost. Second, a major shift in nosocomial infections from gram-positive to gram-negative bacteria occurred in hospital patients during the 1960's and the early 1970's. Therefore, clinical microbiology laboratories, faced with the pressing need for accurate identification systems for enteric bacteria, carried out an extensive characterization of the members of this group of bacteria, including *E*. *coli*.

B. Taxonomy and Characterization

Escherichia coli is a member of the family *Enterobacteriaceae* and has been described by Brenner (1984). *Escherichia* is a Gram-negative rod which can be motile by peritrichous flagella or nonmotile. *Escherichia* is also a facultative anaerobe which has both a respiratory and a fermentative type of metabolism, and commonly occurs in the intestinal tract of humans and other animals.

E. coli K-12 was originally isolated from a convalescent diphtheria patient in 1922 (Bachmann, 1972). Because it lacks virulence characteristics, grows readily on common laboratory media, and has proven to be a valuable tool for microbial physiology and genetics research, it has become the standard bacteriological strain used in microbiological research and teaching. *E. coli* K-12 is now considered an enfeebled organism as a result of being maintained in the laboratory environment for over 70 years (Williams-Smith, 1978).

E. coli can be readily differentiated from closely related bacteria by a number of standard tests. Classically, this has been accomplished by testing for production of indole from tryptophan, production of acid from glucose media using the dye methyl red as an indicator, lack of production of acetoin as a metabolic endproduct (also known as the Voges-Proskauer reaction), and the inability to utilize citrate as a sole source of carbon. Collectively, these reactions are known as the IMViC battery. The IMViC battery was developed for the analysis of water samples where it was important to differentiate *E. coli*, which was found to be always associated with fecal contamination of water, from other closely related bacteria which could be found naturally in water sources. Further refinements of the IMViC tests are used today and are available as commercial test kits.

C. Related Species of Concern

Taxonomically, the four species of the genus *Shigella* are closely related to *E. coli*. *Shigella* species cause diarrhea in humans and are classified as Class 2 agents under the NIH Guidelines. The *Shigella* species and *E. coli* share a high level of DNA sequence homology and many protein and polysaccharide capsular antigens. [These capsular antigens can be used to distinguish between *E. coli* strains and the pattern of capsular antigens determine the organism's "serotype" (Smith 1977).] The two genera can be distinguished based on the fact that *E. coli* has a unique colony morphology when grown on certain differential laboratory media (Jawetz et al., 1987). Commercially prepared kits for distinguishing between these organisms are available.

Most *E. coli* serotypes are benign and may even contribute to normal function and nutrition in the gastrointestinal tract. A few *E. coli* serotypes are pathogens.

E. coli K-12 strains in use today are from standard culture collections (Bachmann, 1972), such as the American Type Culture Collection and are not recent environmental isolates. As a result, these K-12 strains are well-characterized and should be expected to remain as pure cultures under standard microbiological practices. K-12 strains are distinguishable from both *Shigella* and other Escherichia (Cooke, 1974, Orskov 1978, Schmidt 1973).

III. HAZARD ASSESSMENT

The Proceedings of the Falmouth Workshop held in June 1977 served as a primary source for this assessment (Gorbach, 1978).

A. Human Health Hazards

The potential of K-12 strains to present risks to human health are examined in this assessment by analyzing K-12 in terms of (1) the phenotypic traits relevant to colonization of the colon, and (2) toxin production.

1. Colonization and Pathogenicity

E. coli is an inhabitant of the human colon, and it is thought that the primary means through which humans acquire their intestinal flora is through ingestion. Workers in fermentation facilities would not be expected to ingest *E. coli* under standard good practice, which prohibits the ingestion of food in work areas; however, some inhaled bacteria could be swallowed.

In order to evaluate K-12's potential to colonize the human intestine the following should be addressed: (1) the characteristics relevant to *E. coli* colonization of the human colon, and how K-12 compares to other *E. coli* in terms of these traits, and (2) data relevant to colonization potential of K-12 strains.

The binding of an *E. coli* to the mucosal surface of the colon requires two factors. The first factor is the production of a specific glycocalyx or fimbriae from the surface of the bacterium. This specific glycocalyx recognizes a specific lectin on the surface of the enterocyte lining of the human colon. The glycocalyxes appear to bind to structures such as the mucus glycoproteins elaborated from the goblet cells of the intestine. In gram-negative bacteria, the polysaccharide chains arising from the core of the lipopolysaccharides in the outer membrane appear to be the major ones which affect binding to the colon.

E. coli K-12 is defective in at least three cell wall characteristics. The outer membrane has a defective lipopolysaccharide core which affects the attachment of the O-antigen polysaccharide side chains (Curtiss, 1978). Second, it does not have the type of glycocalyx required for attachment to the mucosal surface of the human colon (Edberg, 1991) as a result of the altered O-antigen properties noted above. Finally, K-12 strains do not appear to express capsular (K) antigens, which are heat-labile polysaccharides important for colonization and virulence (Curtiss, 1978).

K-12, thus, is not able to colonize the human intestinal tract under normal conditions, even after ingestion of billions of organisms (Anderson, 1975, Cohen et al., 1979., Levy and Marshall, 1981; Levy et. al, 1980, Smith, 1975). As noted above, K-12 is defective in cell wall components relevant to the ability to recognize and adhere to the mucosal surface of colonic cells (Curtiss, 1978). The normal flora in residence in the colon thus can easily exclude K-12, and prevent it from colonizing the human colon.

A number of experiments have been conducted to measure the ability of K-12 to colonize in comparison to other *E. coli*. These experiments basically fall into two categories. First, those in which the normal human and/or animal flora was substantially reduced in order to provide the best opportunity for K-12 to colonize. The second category, known as co-colonization experiments, were to determine which strains could out-compete which other strains (Anderson, Gillespie and Richmond 1973; Anderson 1974; Burton et al. 1974; Anderson 1975; Smith 1975; Freter 1978; Laux, Cabelli and Cohen 1982; Myhal, Laux and Cohen 1982; Levine et al. 1983).

For the first category of experiments, normal flora were reduced through treatment with antibiotics or the experiments were performed using germ-free mice. In the second category, normal flora were reduced and two strains of *E. coli* were introduced to determine whether one strain could establish itself at the expense of the other.

These sets of experiments indicate that K-12 is a poor colonizer. Most strains of *E. coli*, including K-12, can colonize the intestine when the normal flora is substantially reduced. K-12 thus can colonize individuals whose normal intestinal flora is reduced through antibiotic therapy or is affected by other variables that can affect colonization such as anti-motility drugs. Similarly, *E. coli* K-12 can colonize germ-free mice, but is quickly displaced when the mice are fed other *E. coli* (Curtiss, 1978). In co-colonization experiments, *E. coli* K-12 has consistently been outcompeted by other *E. coli*. These studies indicate that K-12 is a poor colonizer and that indigenous intestinal microorganisms have a large competitive advantage over K-12 strains.

Aside from K-12's inherent decreased ability to colonize the gut, the culture conditions under which a K-12 strain is grown decrease the ability of the strain to colonize the gastrointestinal tract. Indeed, organisms grown under laboratory or fermentation conditions in general are not particularly competitive in comparison to microorganisms isolated from the environmental niche of the organism. Similarly, *E. coli*, which had been grown in continuous-flow cultures, were poor colonizers in mice with a normal cecal flora (Freter et al. 1983).

The medium in which *E. coli* are introduced to the GI tract is also important. Even in germ-free mice, the survival of K-12 strains is minimal unless the cultures of bacteria are introduced in a basic buffer such as bicarbonate, since K-12 strains are killed by stomach acidity (Freter, et al., 1983).

These two considerations further reduce the probability that K-12 strains grown under commercial fermentation conditions will be able to colonize the GI tract of workers. Such cultures would not be metabolically adapted to the human gut, and the microorganisms would not usually be grown in basically buffered fermentation media.

In order to cause disease, a microorganism must colonize a site of the human body and express virulence characteristics. On the basis of colonization alone, *E. coli* K-12 is innately defective as a pathogen, and a very low likelihood of acting as a pathogen of humans or animals.

The ability of certain *E. coli* serotypes to cause disease appears to be associated with certain specific capsular antigens. These serotypes of *E. coli* cause diarrhea, urinary tract infection, bacteremia and neonatal meningitis (Silverblatt and Weinstein 1979). It appears that colonization of the colon by the pathogenic serotype is a precondition for these illnesses (Moxon, Glode, Sutton 1977; Silverblatt and Weinstein 1979). The likelihood of these types of infections occurring with K-12 is thus very low for two reasons. First, since *E. coli* K-12 is a very poor colonizer it is unlikely to establish itself in the colon. Second, K-12 lacks adhesion and other virulence factors necessary for pathogenesis (Curtiss, 1978, Gorbach 1978, Edberg, 1991).

2. Toxin Production

A number of toxins affecting humans have been identified in *E. coli*: shiga-like toxins (SLT-I and II) and heat labile and heat stable toxins. The related genus *Shigella* produces shiga toxin (ST). These toxins are factors through which pathogenic serotypes cause diarrhea.

E. coli K-12 appears to lack the ability to produce significant quantities of toxins that affect humans (Edberg, 1991). There are two studies on production of toxins in K-12 strains. O'Brien and Holmes (1987) state that all strains of *E. coli* K-12 examined produced low levels of SLT as detected by monoclonal and polyclonal antibodies; however, DNA probes for SLT-I did not hybridize with DNA from these strains. These conflicting results are still unexplained. Edberg (1992) noted that strain K-12 was found to be in the low level toxin producing category ($2X10^2$ - $6X10^2$ CD50 per ml of sonic lysate). However, it is unclear whether this cell line-based assay is measuring an actual toxin or a protein that may exert activity against the cell.

No records of K-12 enterotoxin-induced disease for fermentation workers were located in the literature. This is not surprising since the K-12 would have to colonize and invade the tissue of the GI tract of workers in order to deliver the toxin to its site of action.

3. Conclusions

E. coli K-12 is not considered a human or animal pathogen nor is it toxicogenic. Any concerns for *E. coli* K-12 in terms of health considerations are mitigated by its poor ability to colonize the colon and establish infections.

B. Environmental Hazards

1. Hazards to Animals

A number of experiments with *E. coli* K-12 have been conducted in animal models (Burton et al. 1974; Freter, *et al.*, 1983; Cohen and Laux, 1985; Laux *et al*. 1982; Myhal *et al*.).

These experiments demonstrate that E. coli K-12 will not under normal conditions colonize the GI tract of animals. A number of researchers have tried in vain to implant K-12 in the GI tract of laboratory animals under normal conditions (Freter 1978, Curtiss 1978). Negative results have been noted in mice, pigs, chickens, pigs and calves. K-12 is also unlikely to behave as a pathogen (Gorbach 1978).

2. Hazards to Plants

There are no data that suggest that *E. coli* K-12 strains have adverse effects on plants. The genus *Escherichia* is not considered a plant pest species by the U.S. Department of Agriculture (7 CFR 330, et seq.).

3. Hazards Posed to Other Microorganisms

No evidence exists relative to hazardous effects of *E. coli* K-12 strains on other microorganisms in the environment (Sayre, 1991).

4. Conclusions

E. coli K-12 strains are very unlikely to pose a hazard to either animals, plants, or other microorganisms.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

E. coli K-12 is considered an exempt host system under the NIH Guidelines. This microorganism also falls under the Class 1 Containment under the European Federation of Biotechnology guidelines (Frommer et al., 1989).

No data were available for assessing the release and survival specifically for fermentation facilities using E. coli K-12. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, ie. near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

The natural habitat of *E. coli* is the large bowel of mammals. However, *E. coli* K-12 has lost the ability to colonize the gut and cannot survive in the bowel for very long. The ability of *E. coli* to survive under environmental conditions is also limited. In one study, *E. coli* HB101 introduced into non-sterile soil in saline declined to levels below detection (1-20 cells/g soil) after 21 days. *E. coli* also declines rapidly in seawater. There are of course many factors that determine the survival of these organisms in the environment and it is unlikely that all the introduced *E. coli* would die. However, it is reasonable to assume that over the long term, populations of *E. coli* K-12 in soil would be very low. *E. coli* released to air would not be expected to survive well because of the low nutrient levels and drying conditions (LaVeck, 1991).

2. Releases

Estimates of the number of *E. coli* K-12 organisms released per production batch are tabulated in Table 1. The minimally controlled scenario assumes no treatment of the fermentor off-gas and assumes 100-fold (2 log) reduction of the maximum cell density of the fermentation broth resulting from inactivation (Reilly, 1991). The containment criteria required for the full exemption scenario assume the use of in-line filters to treat vent gases and a 99% removal efficiency under normal operating conditions. They also assume an overall 6-log reduction relative to the maximum cell density of the fermentation broth resulting from inactivation steps (Reilly, 1991).

TABLE 1. Estimated Number of Viable E. coli K-12 Organisms Per Production Batch

Release Media	Minimally Controlled (cfu/day)	Full Exemption (cfu/day)	Release (days/year)
Air Vents	$2x10^8$ - $1x10^{11}$	$2x10^6$ - $1x10^9$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{13}$	$7x10^9$	90
Soil/Landfill	$7x10^{15}$	$7x10^{11}$	90

Source: Reilly, 1991

3. Air

Exposure to viable E. coli K-12 in the atmosphere is expected to be minimal because of the poor survival and high dilution in the atmosphere (LaVeck, 1991).

4. Water

Surface water concentrations of organisms were estimated using the 10% and 50% flow values for SIC code 283 (drug, medicinal chemicals, pharmaceuticals) that release to surface water. The SIC code flow was estimated using 128 indirect dischargers (facilities that send their waste to a POTW) and direct dischargers (facilities that have an NPDES permit to discharge to surface water). Discharger data were extracted from the IFD (Industrial Facilities Dischargers) database and surface water flow data were taken from the RXGAGE database, maintained by the EPA. These data, indicating 2 to 4 log reduction in cfu release per day (Table 2), were partitioned into percentile rankings and flows for the 10th percentile (small river) and 50th (average river) were extracted and used for the exposure calculations. Flow is expressed in Millions of Liters/Day (MLD). Mean Flow is the average flow value, and 7Q10 flow is the lowest flow observed over 7 consecutive days during a 10-year period. Concentrations of microorganisms in surface water are calculated for both the minimally controlled and the full exemption scenarios. The surface water concentrations assume 100% survival of the organism during treatment. While this survival level may exist for a short period of time, it is expected that the number of viable organisms will decrease rapidly over a 30-day period. Escherichia coli K-12 would also be expected to survive at low levels in POTW sludge.

TABLE 2. E. coli K-12 Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	Q710	Mean	Q710
Minimally Controlled				
10th Percentile	159	4.57	$4.4x10^5$	$1.53x10^7$
50th Percentile	768	68.13	$9.11x10^4$	$1.03x10^6$
Full Exemption				
10th Percentile	159	4.57	$4.4x10^1$	$1.53x10^3$
50th Percentile	768	68.13	$9.11x10^0$	$1.03x10^2$

*MLD = million liters per day
Source: LaVeck, 1991

5. Soil

Since E. coli is not a normal inhabitant of the soil, its survival would not be expected under these conditions. According to LaVeck (1991), E. coli HB101 introduced into non-sterile soil in saline declined to levels below detection (1-20 cells/g soil) after 21 days. Based on this study, and other studies cited in the section on environmental hazards and LaVeck (1991), survival of various strains of E. coli could range from 0 - 10^4 cells/gram of soil.

V. INTEGRATION OF RISK

A. Discussion

E. coli K-12 is a well-studied bacterium which has been the subject of extensive research in microbial physiology and genetics and commercially exploited for a variety of industrial uses. The natural habitat of the parent species, *E. coli,* is the large bowel of mammals. *E. coli* K-12 has a history of safe use. Its derivatives are currently used in a large number of industrial applications, including the production of specialty chemicals (e.g., L-aspartic, inosinic, and adenylic acids) and human drugs such as insulin and somatostatin. An insulin-like hormone for use as a component of cell culture media, resulting from a fermentation application in which *E. coli* was used as the recipient, has already been reviewed under TSCA (Premanufacture Notice P87-693).

E. coli K-12 was originally isolated from a convalescent diphtheria patient in 1922 (Bachmann, 1972). Because it lacks virulence characteristics, grows readily on common laboratory media, and has proven to be a valuable tool for microbial physiology and genetics research, it has become the standard bacteriological strain used in microbiological research and teaching. *E. coli* K-12 is now considered an enfeebled organism as a result of being maintained in the laboratory environment for over 70 years (Williams-Smith, 1978). As a result, K-12 strains are unable to colonize the intestines of humans and other animals under normal conditions.

E. coli K-12 strains are not likely to pose a risk to human or animal health, to plants, or to other microorganisms. *E. coli* K-12 has been utilized for 70 years, often in industrial settings with high volumes and cell densities. Moreover, *E. coli* K-12 has been employed extensively in research laboratories. In the industrial setting with the use of appropriate industrial practices for handling microorganisms, and with good laboratory practices in the research setting, the potential for K-12 strains to colonize the human colon is quite low.

Likewise, the ecological risks associated with the use of *E*. *coli* K-12 are low. Industrial fermentation uses of this organism are expected to result in a low number of microorganisms released from the fermentation facility. Given its natural habitat of the large bowel of mammals, *E*. *coli* will not likely survive for long periods in soil, water, or air. *E*. *coli* K-12 has lost the ability to colonize the gut and has been shown to have poorer survival characteristics in soil and water than other *E*. *coli*. The ability of *E*. *coli* to survive under environmental conditions is thus very limited. *E*. *coli* K-12 has no known survival mechanisms in the environment, such as the ability to produce spores.

In conclusion, the use of *E*. *coli* K-12 under contained conditions in fermentation facilities present low risk.

B. Recommendations

E. *coli* K-12 is recommended for the tiered exemption.

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